Exhibit 2.

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Determination of genetic diversity within the genus *Bifidobacterium* and estimation of chromosomal size

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Abstract

Pulsed-field gel electrophoresis was proven to be an efficient means of differentiating 25 strains of *Bifidobacterium* obtained from culture collections. *Xba*I, *Spe*I, *Dra*I restriction enzyme profiles indicated genomic heterogeneity among strains. When seven human isolates of bifidobacteria were compared using the same methods, two individual banding patterns were obtained. However, despite its discriminatory potential, pulsed-field gel electrophoresis was shown to be of no value in taxonomic identification. Genomic sizes estimated for eight *Bifidobacterium* strains ranged from 1.5 Mb to 2.1 Mb.

Keywords: *Bifidobacterium*; Pulsed-field gel electrophoresis; Chromosome size

1. Introduction

The genus *Bifidobacterium* is among the three most prevalent bacterial genera in the human colon. According to the most recent classification the genus consists of 32 species, 12 of which are of human origin [1]. Differentiation of species within this genus has traditionally relied on DNA-DNA homology or various phenotypic characteristics [2]. The reputed therapeutic value of these microorganisms has resulted in their incorporation into many functional foods. Because of this, considerable effort has since been devoted to the application of various molecular techniques for the rapid identification of strains. These include the use of genus-specific [3], species-specific [4] and strain-specific [5] probes based on appropriate 16S rRNA sequences. rRNA gene restriction patterns or randomly cloned DNA fragments as species-specific DNA probes have also been used to distinguish between species [6,7].

The technique of pulsed-field gel electrophoresis (PFGE) has been exploited to successfully unravel the organisation of many bacterial genomes revealing the presence of multiple chromosomes, linear chromosomes or large plasmids in different hosts. Rare cutting enzymes employed in conjunction with PFGE have allowed species identification and strain classification within the same species and have also provided useful data for estimating genome size and for genome mapping [8]. Until recently, information on genomic organisation of bifidobacteria was limited to one species of the genus which described intra-species polymorphisms between four of five *B.*

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breve strains examined [9]. However, Roy et al. [10] provided a more extensive study, in which PFGE was used to compare a bank of dairy-related bifidobacteria comprising culture collection and commercial strains. The method was successful in distinguishing between strains even within a given species and in establishing the origin of commercial strains.

The aim of this study was to employ PFGE to (i) determine intra- and inter-species genetic diversity of a number of culture collection typed strains of bifidobacteria including representatives of the four prevalent human species, (ii) evaluate the technique of PFGE as a means of differentiating between and speciating a range of human bifidobacterial isolates, and (iii) estimate genomic sizes for representative strains.

2. Materials and methods

2.1. Bacterial strains

Bifidobacteria strains studied are listed in Table 1. Bifidobacteria were routinely cultured in TPY broth [11] at 37°C under anaerobic conditions which were maintained using the anaerobic Gas Pak system (Merck, Darmstadt, Germany) in an anaerobic chamber.

Table 1
Bifidobacterium strains

Species	Strain	Source
B. bifidum	NCFB 1452	Nursling stools
	NCFB 1453	Nursling stools
	NCFB 1454	Nursling stools
	NCFB 1455	Nursling stools
	NCFB 1456	Nursling stoold
	NCFB 2203	Infant intestine
	NCIMB 8810	Nursling stools
	Chr. Hansens 12	Commercial strain
B. infantis	NCFB 2255	Infant intestine
	NCFB 2256	Infant intestine
	NCFB 2205	Infant intestine
	Visby 420	Commercial strain
B. breve	NCFB 2257	Infant intestine
	NCFB 2258	Infant intestine
	NCIMB 8815	Nursling stools
	NCIMB 8807	Nursling stools
	NCTC 11815	Infant intestine
B. adolescentis	NCFB 2204	Adult intestine
	NCFB 2229	Adult intestine
	NCFB 2230	Adult intestine
	NCFB 2231	Adult intestine
	NCTC 11814	Adult intestine
Bifidobacterium sp.	UCC 35612	Adult intestine
	UCC 35624	Adult intestine
	UCC 35658	Adult intestine
	UCC 35652	Adult intestine
	UCC 35675	Adult intestine
	UCC 35678	Adult intestine
	UCC 35687	Adult intestine
B. angulatum	NCFB 2236	Human faeces
B. catenulatum	NCFB 2246	Human faeces
B. pseudocatenulatum	NCIMB 8811	Nursling stools

NCFB: National Collection of Food Bacteria, Reading, UK. NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, UK. NCTC: National Culture Type Collection, London, UK. UCC: Culture Collection, University College, Cork, Ireland. Visby: Laboratorium Wiesby, Germany. Chr. Hansens Laboratory A/S, Copenhagen, Denmark.

2.2. Preparation of bacterial plugs

Agarose plugs containing genomic DNA were prepared as follows: 100 ml volumes of TPY were inoculated (2%) with the appropriate microorganism. When an OD_{600nm} of ~1 was attained the cells were harvested, washed once in 50 mM EDTA, pH 8.5 and finally resuspended in 1.5 ml of the same solution. 500 µl of this cell suspension was heated to 42°C and mixed with 3 ml of molten 1% low melting point agarose and the resulting solution was poured into the mould chamber (Bio-Rad, Richmond, CA, USA). Solidified blocks were incubated for 4–16 h in lysozyme solution (2 mg ml^{-1} lysozyme, 0.05% *N*-lauryl sarcosine in 50 mM EDTA, pH 8.5). The blocks were then heated overnight (12–16 h) with proteinase K at 42°C (2 mg ml^{-1} proteinase K, 1% SDS, 0.1 M Tris in 50 mM EDTA, pH 8.5). Inserts were washed at least three times with 50 mM EDTA, pH 8.5 at room temperature with gentle shaking and were finally stored in the same solution at 4°C.

2.3. Restriction digestion of DNA in agarose blocks

Agarose blocks were cut into 1×2×5 mm segments with a scalpel and washed in 1 ml sdH_2O on ice for 15 min to lower the EDTA concentration in the plug. The water was replaced with 100–200 µl restriction endonuclease buffer and left to equilibrate overnight at 4°C. The buffer was replaced and the

appropriate restriction endonuclease added. Digestion was performed at the recommended temperature for the chosen enzyme.

2.4. PFGE

Separation of DNA fragments was performed in a CHEF DR II apparatus (Bio-Rad). Agarose gels were prepared using 1% pulsed-field certified agarose (Bio-Rad) in 0.5×TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). Electrophoresis was performed at 8°C with pulse times varying with the size range of DNA fragments. To resolve fragments in the size range 150–600 kb switch times of 6–60 s were used, for 40–200 kb fragments switch times of 1–20 s, and for fragments of 20–45 kb switch times varied between 0.1 and 5 s. The sizes of the restriction fragments were determined by comparison with standard size markers. The standards used were Lambda ladder PFG Marker (48.5–1018.5 kb), Yeast Chromosome PFG Marker (225–1900 kb) and Low Range Marker (0.13–194 kb), all obtained from New England Biolabs (Beverly, MA). Gels were ethidium bromide-stained and photographed on a UV transilluminator using either a Polaroid MP4 Land camera containing type 667 film or a UVP image store 5000 Gel Documentation System linked to a Sony video graphic printer.

3. Results and discussion

Molecular technologies are currently the favoured methods for discriminating between strains of many different bacterial genera. In general, the available literature indicates that even though it is more time-consuming and labour-intensive, for many genera PFGE is more effective than ribotyping, SDS-PAGE or random amplified polymorphic DNA (RAPD) PCR assays in discriminating between strains [12]. Because of this PFGE was chosen in this study as a means of distinguishing between bifidobacterial strains. In this study 25 typed bifidobacterial strains representing the four predominant species present in the human intestine were first digested with restriction enzymes and resultant profiles compared. These included *B. bifidum* (8 strains), *B. adolescentis* (5 strains), *B. infantis* (4 strains), *B. breve* (5 strains) and three other strains (*B. angulatum*, *B. catenulatum*, *B. pseudocatenulatum*) of species less frequently isolated from humans. In addition seven human isolates were examined in a similar manner.

3.1. Selection of suitable enzymes for PFGE analysis of Bifidobacterium species

Physical genome analyses require restriction enzymes that cut the bacterial genome into a limited number of fragments. Due to the high GC content of bifidobacteria (55–64%) enzymes incorporating AT-rich sequence (*Dra*I, *Ase*I, *Ssp*I) or 8 bp sequence (*Sfi*I, *Not*I, *Pac*I, *Swa*I, *Ase*I) in their recognition sites or possessing the tetranucleotide CTAG in their restriction sites (*Xba*I, *Spe*I, *Avr*II) were tested, as these would be expected to cleave such genomes infrequently [13]. Only *Ase*I, *Spe*I and *Xba*I proved to be suitable in that they produced fewer than 30 bands. *Dra*I was a very effective rare cutter for *Bifidobacterium* sp. 35612 but continuously resulted in partial digests for a number of other strains. Employing different switch times enabled good resolution of fragments of all molecular masses.

3.2. Comparison of genomic restriction digest patterns of culture collection strains

It was of interest to study whether strains within the same species could be differentiated by PFGE. Initially the 25 culture collection strains were examined. These included some 'type' strains which, although obtained from different culture collections, are believed to be identical. Of the five *B. breve* strains analysed some appeared to be genetically similar (Fig. 1). The type strains 2257 and 11815 which are listed in the culture collections as being synonymous appeared indistinguishable when digested with *Xba*I (Fig. 1, lanes 1 and 5) and when digested with *Spe*I, profiles differed by only one band. Strains 8815 and 8807, which according to collection catalogues listings are phenotypically different, were shown to display very related patterns. *Xba*I macrorestriction analysis of these strains resulted in profiles with very minor differences (one obvious band; Fig. 1, lanes 3 and 4). When five strains of the species *B. adolescentis* were examined in a similar manner they also appeared genetically quite homologous, and

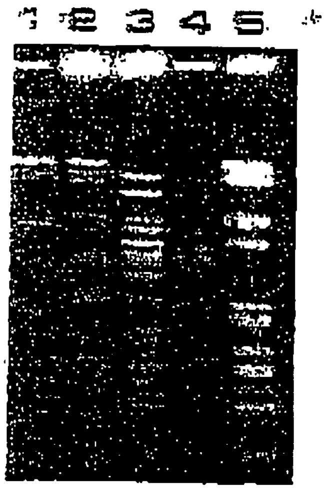

Fig. 1. *Xba*I macrorestriction profiles of five *B. breve* strains. PFGE was performed at 180 V for 24 h with a ramped switch time of 2–12 s. Lane 1, *B. breve* 2257; lane 2, *B. breve* 2258; lane 3, *B. breve* 8815; lane 4, *B. breve* 8807; lane 5, *B. breve* 11815.

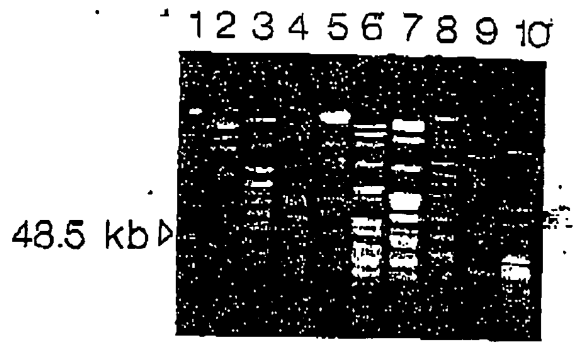

Fig. 2. Total DNA digested with *Spe*I. Gels were resolved at 180 V for 24 h with a ramped switch time of 2–12 s. Lane 1, low range PFG marker; lane 2, *B. bifidum* Chr. Hansens 12; lane 3, *B. catenulatum* 2236; lane 4, *B. pseudocatenulatum* 8811; lane 5, *B. bifidum* 2203; lane 6, *B. bifidum* 1456; lane 7, *B. bifidum* 1455; lane 8, *B. bifidum* 1454; lane 9, *B. bifidum* 1453; lane 10, *B. bifidum* 1452.

again the type strains 11814 and 2204 produced the expected identical patterns when digested with *Xba*I, *Spe*I and *Dra*I (data not shown) reiterating the reproducibility of this method. The seven strains representing the species *B. bifidum* exhibited a greater degree of genomic heterogeneity with both *Xba*I and *Spe*I restriction digests (Fig. 2). The profiles obtained in this study for strain 2203 appeared to correlate well with *Xba*I/*Spe*I digests of the same strain (ATCC 15696) reported by Roy et al. [10]. The type strains 8810 and 1454 displayed identical patterns (data not shown) but in general the strains within this species appeared quite diverse. When strains of this and other species were compared, inter-species differences were not found to be any more pronounced than intra-species differences (Fig. 2). In fact, *B. pseudocatenulatum* 8811 and *B. bifidum* 1455 displayed identical patterns (Fig. 2, lanes 4 and 7). These strains, obtained from different culture collections, are believed to be phenotypically identical although speciated differently. While four strains from the species *B. infantis* were compared, all produced different patterns when digested with *Xba*I and *Spe*I (*Xba*I digests of three strains shown in Fig. 3, lanes 5, 6, 7). Intra-species relatedness did not appear to be greater than inter-species relatedness, e.g. when digested with the same enzyme, the three largest *B. infantis* 2256 fragments co-migrated with three of the four largest *B. pseudocatenulatum* 8811 fragments, only one of which co-migrated with fragments from the related species *B. catenulatum* (Fig. 3, lanes 6, 3, 4). However, over a range of digests *B. catenulatum* and *B. pseudocatenulatum* were shown to consistently result in a greater number of co-migrating bands. DNA-DNA homology studies have indicated that the species *B. catenulatum* and *B. pseudocatenulatum* are very similar in that they exhibit degrees of homology as high as 75% [2]. In this study the similarity of macrorestriction

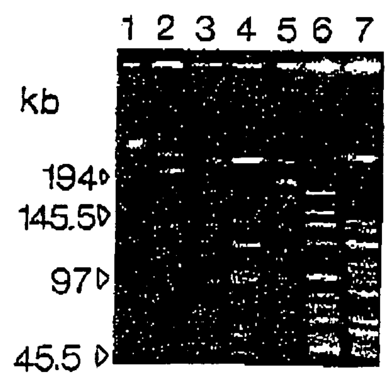

Fig. 3. *Xba*I digests of total DNA from *Bifidobacterium* strains. Gels were run at 180 V for 24 h with a ramped switch time of 2–10 s. Lane 1, low range PFG marker; lane 2, *B. angulatum* NCFB 2236; lane 3, *B. pseudocatenulatum* NCIMB 8811; lane 4, *B. catenulatum* NCFB 2246; lane 5, *B. infantis* Visby 420; lane 6, *B. infantis* NCFB 2256; lane 7, *B. infantis* NCFB 2255.

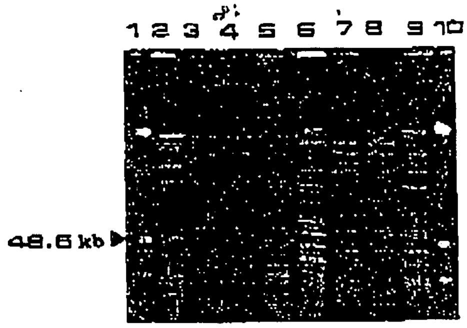

Fig. 4. Comparison of *AseI* and *AscI* digests of human bifidobacteria isolates. PFGE was performed at 180 V for 24 h with a ramped switch time of 3–5 s. Lane 1, low range PFG marker; lane 2, *B. bifidum* 2203 (*AseI*); lane 3, *Bifidobacterium* isolate 35612 (*AseI*); lane 4, *Bifidobacterium* isolate 35624 (*AseI*); lane 5, *Bifidobacterium* isolate 35658 (*AseI*); lane 6, *B. bifidum* 2203 (*AscI*); lane 7, *Bifidobacterium* isolate 35612 (*AscI*); lane 8, *Bifidobacterium* isolate 35624 (*AscI*); lane 9, *Bifidobacterium* isolate 35658 (*AscI*), lane 10, low range PFG marker.

patterns obtained for 1455 and 8811 with those obtained for *B. catenulatum* suggest that these strains are indeed better classified as *B. pseudocatenulatum*.

3.3. Comparison of genomic patterns of human isolates

A bank of seven human intestinal isolates, classified as members of the genus *Bifidobacterium* both by their 3:2 acetate:lactate ratio as determined by HPLC and by classical positive fructose 6-phosphate phosphoketolase reactions, were also compared by PFGE. As these particular strains were all isolated from one individual it was of interest to determine if they were identical. Therefore the strains were compared by restricting their genomic DNA with a number of enzymes (*Avr*II, *Dra*I, *Ase*I, *Xba*I, *Spe*I, *Not*I). Only two classes of restriction patterns were obtained with isolate 35658 displaying a different banding pattern to each of the other isolates. The two types of banding patterns generated for *Ase*I and *Asc*I are shown in Fig. 4 (lanes 3–5 and 7–9, respectively).

The patterns obtained from the two human isolate groups (represented by 35658 and 35612) were compared to digest patterns of a number of culture collection strains (*B. adolescentis, B. angulatum, B. bifidum, B. infantis, B. longum, B. pseudocatenulatum* and *B. breve*) in an attempt to identify the isolates to species level. However, although co-migrating bands could be identified, due to the extreme sensitivity of this method, species boundaries could not clearly be defined. It would therefore appear that although PFGE is a good discriminatory technique it would require a great deal of screening to establish its taxonomic relevance.

3.4. Genomic sizes

Genome sizes were estimated for eight strains representing different species (*Bifidobacterium* sp. 35612, *B. angulatum, B. catenulatum, B. pseudocatenulatum, B. bifidum, B. breve, B. infantis, B. adolescentis*; see Table 2). Fragments obtained from at least two different enzymes were sized. In addition, each gel was run under three different ramped switch times as described in Section 2 to obtain maximum resolution of different molecular mass fragments. In each case the size of the restriction fragments was determined as described by Heath et al. using two flanking size standards [14]. The presence of multiple bands was assessed by visual evaluation of ethidium bromide staining. Estimated genome sizes ranged from 1.5 Mb to 2.1 Mb with an average genome size of 1.8 Mb. This value places them in the lower category size range for bacterial chromosomes, smaller than

Table 2
Genome restriction analysis of *Bifidobacterium* strains by PFGE

Strain	Enzyme	Total number of restriction fragments	Genome size (Mb)
Bifidobacterium sp. 35612	*Xba*I	18	1.85
	*Spe*I	21	1.5
	*Dra*I	11	1.79
B. angulatum	*Xba*I	16	1.77
	*Spe*I	13	1.50
B. catenulatum	*Xba*I	18	1.46
	*Spe*I	18	1.69
B. pseudocatenulatum	*Xba*I	19	1.65
	*Spe*I	19	1.55
B. bifidum 8810	*Xba*I	16	1.96
	*Spe*I	25	1.80
B. breve 2257	*Xba*I	11	1.53
	*Spe*I	21	1.64
B. infantis 2255	*Xba*I	18	1.67
	*Spe*I	23	2.07
B. adolescentis 2231	*Xba*I	16	1.97
	*Spe*I	19	2.02

the genetically related Actinomycetaceae, *Streptomyces* sp. (6.5–8.2 Mb) and *Corynebacterium glutamicum* (2.987 Mb) [15]. PFGE although a popular method of chromosome sizing can at best only be relied on to give an estimated value. The values obtained for *B. breve* NCFB 2257 is very close to the 1.6 Mb value proposed by Roy et al. [10] for the synonymous strain *B. breve* ATCC 15700 but lower than the 2.1 Mb values reported by others for members of the same species [9]. Profiles obtained for *B. infantis* strain NCFB 2205, when compared to those described previously [10] for the equivalent strain ATCC 15697, revealed some slight differences. In this study a greater number of smaller sized bands resulted in a higher genomic size of approximately 1.87 Mb compared to the 1.5 Mb reported by Roy et al. [10]. This study is also the first to report estimated genome sizes for strains of *B. angulatum*, *B. catenulatum* or *B. pseudocatenulatum*.

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